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The Development of a Direct Homologous Radioimmunoassay for Serum Cortisol [1], [2]

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Summary: This article describes the synthesis of a pure cortisol-3-(O-carboxymethyl) oxime with its subsequent use in producing highly specific antibodies and a ^{125}I-tracer with a shelf life in excess of several weeks.

An assay was developed using a pH of 4, thus allowing a direct measurement of cortisol in serum without extraction or heat denaturation.

The quality-control parameters were checked – the intra-assay C.V. in the working range (area of clinical interest) was under 5%, and the interassay C.V. under 11%. Correlation studies with results using other antisera and with ^{3}H-tracer are highly significant with the slope of the regression curve deviating less than 2% from the ideal volume.

Finally the assay is fully automatable and is suitable for most automatic pipetting and analysis systems.

Die Entwicklung eines direkten homologen Radioimmunoassays für Serum-Cortisol

Zusammenfassung: Diese Arbeit beschreibt die Herstellung eines reinen Cortisol-3-(O-carboxymethyl) oxims und seine weitere Verwendung zur Gewinnung hochspezifischer Antiseren und eines ^{125}I-Tracers mit einer Einsatzmöglichkeit von mehreren Wochen.

Es wurde ein Assay entwickelt, der bei pH = 4 arbeitet. Dies erlaubt die direkte Bestimmung von Serum-Cortisol ohne Extraktion oder Hitzedenaturierung.

Als Qualitätskontrollgrößen fanden sich ein Intraassay-Variationskoeffizient im steilen Bereich der Standardkurve, der für klinische Fragestellungen wichtig ist, von unter 5%, und ein Interassay-Variationskoeffizient von unter 11%. Die Korrelationsstudien mit Ergebnissen, die man mit anderen Antiseren bzw. mit einem ^{3}H-Tracer erhielt, waren alle hochsignifikant mit einer Abweichung der Regressionsgeraden von weniger als 2%. Der Assay ist voll automatisierbar und für die meisten automatischen Pipettier- und Analysensysteme adaptierbar.

Introduction

As the request for the routine measurement of the cortisol concentration in serum grows, so does the need for a simple and accurate method with high capacity. The method of choice is an immunoassay.

Because of its low molecular weight, cortisol does not have any intrinsic antigenic properties (1) and must therefore be coupled to a carrier protein to form a complex capable of producing an antibody response. Because of the large number of naturally occurring

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steroids, many of which are similar in structure to cortisol, a specific assay is needed which will measure cortisol alone in serum. This makes the synthesis of pure specific defined derivatives of the utmost importance.

Another important point is the choice of isotope for the radioactive labelling. Because of the lower costs, higher specific activity and counting efficiency, not to mention the relative case of aqueous waste disposal, a ^{125}iodine-tracer is the label of choice.

Finally, in order to achieve a high throughput of samples, an extractive or heat denaturation step to free cortisol from cortisol binding globulin must be avoided in order to allow automation of the method.

These problems have been dealt with individually in the construction of the assay described here.

Materials and Methodology

All chemicals were of "pro-analysi" grade or "puriss" grade. The following chemicals were purchased from Merck (Darmstadt):

Cortisol, all reagents for buffer preparation, all reagents used as solvents, molecular sieve (0.4 nm), Silica Thin Layer Chromatography plates, Tyramine hydrochloride, Tyrosine-methyl-ester hydrochloride, Chloramine-T, Sodium metabisulphite, Potassium bromide, Polyethylene glycol 6000, N-ethyl-N(3-dimethylaminopropyl) carbodiimide hydrochloride.

Human serum albumin, β-, γ-globulin and thyroglobulin were purchased from Sigma Chemical Co. (Taufkirchen)

Rabbit-γ-Globulin was purchased from Serva (Heidelberg)

Carboxymethoxylamine hemihydrochloride was purchased from EGA-Chemie (Frankfurt/Main)

Pertussis vaccine and *Freund*'s Complete Adjuvant were obtained from Behringwerke (Marburg/Lahn)

Sodium^{125}iodide was obtained from Amersham-Buchler (Braunschweig)

Cortisone was obtained from Schering (Berlin)

Fludrocortisone and Triamcinolone were purchased from van Heyden (Munich)

Betamethasone was obtained from Byk-Essex (Munich)

Methylprednisolone was obtained from Homburg (Frankfurt/Main)

Spironolactone was obtained from Boehringer (Mannheim)

All other steroids were purchased from Fluka (Neu-Ulm)

Synthesis of a pure cortisol-3-(O-carboxymethyl)oxime

The principle of the reaction between cortisol and carboxymethoxylamine hemihydrochloride is the conversion of the ketogroups at position C_3 and C_{20} to an oxime group, whose carboxyl group is then available for coupling reactions as used in the production of immunogen and tracer. Three reaction products are formed, namely a C_3-oxime, a C_{20}-oxime and a $C_{3\text{-}20}$-dioxime. The following method describes the production of a pure C_3-oxime.

The following reagents are added to a Quickfit-flask: 1 mmol cortisol (362.5 mg) dissolved in 1 ml dry pyridine, followed by 0.5 mmol carboxymethoxylamine hemihydrochloride (109 mg) dissolved in 1 ml dry pyridine.

An excess of a molecular sieve (0.4 nm) to remove the water formed in the reaction was added and the flask connected to a desiccator-U-tube filled with silica gel and left to react at room temperature.

The reaction was monitored by removing a small volume of the reaction mixture at regular intervals, transferring it to a thin-layer plate (0.2 mm silica gel) and running it in an ethyl acetate, acetic acid, water (9 + 1 + 0.1 by volume) mixture. Ultraviolet light (254 nm) was used to visualise the spots. As time progressed, the spot given by cortisol disappeared, while at the same time a new, more polar spot appeared. The reaction time for the complete disappearance of cortisol was about 6 hours. Figure 1 shows the reaction "catalysed" by pyridine.

The pyridine was removed from the reaction products by using rotary vacuum distillation and the product dissolved in acidified (acetic acid) dioxane. The dioxane was then removed in a similar fashion. The latter process was repeated until no more pyridine could be detected by thin-layer chromatography in ultraviolet light.

The product so obtained consisted mainly of cortisol-3-(O-carboxymethyl)oxime with traces of cortisol-20-(O-carboxymethyl) oxime and C-3,20-di(O-carboxymethyl)oxime together with unchanged cortisol. This mixture was dissolved in 1 ml acidified (acetic acid) dioxane and transferred to a preparative silica gel thin-layer chromatography plate (2 mm) using the same solvent system as above.

Each plate could accommodate ca. 0.1 mmol mixture. After the plates had been run, they were dried and the bands marked under ultraviolet light. It is known from infra-red spectroscopy that the cortisol-3-(O-carboxymethyl)oxime derivate followed the unchanged cortisol. The cortisol-3-(O-carboxymethyl)oxime was scratched from the plate and dissolved from the silica gel by alternate washing with dioxane and solvent mixture, keeping the silica gel in place on a glass sinter and allowing the solvents to flow over it. After removal of the solvent, the product was dried over P_2O_5 in oil-vacuum.

The yield was between 60 and 70% of the theoretical value. The cortisol-3-(O-carboxymethyl)oxime was kept dry and stored at - 20 °C.

Antigen synthesis

Due to favourable experience using bovine thyroglobulin as a hapten carrier for raising antisera to low molecular weight peptides (2), this protein was chosen as the vehicle for coupling to cortisol-3-(O-carboxymethyl)oxime. Two coupling methods were used, namely a carbodiimide (3) and a mixed anhydride reaction (4), to study the molar incorporation and antigenity of the synthesized conjugates.

Carbodiimide method

10 μmol cortisol-3-(O-carboxymethyl)oxime (4.4 mg) were dissolved in 1 ml dioxane to which 1 ml acidified water (pH 5.5) was slowly added dropwise. 60 mg N-ethyl-carbodiimide hydro-

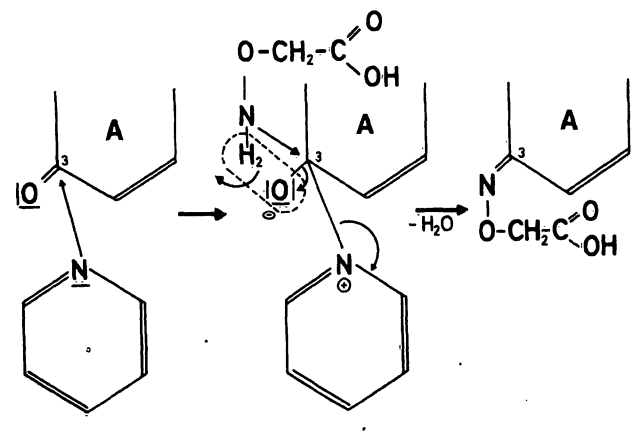

Fig. 1. Pyridine-catalysis of cortisol-3-(O-carboxymethyl)oxime reaction. Only the A-ring and 3-position of the cortisol-molecule is shown.

chloride was added as dry substance and 0.1 μmol bovine thyroglobulin subunit (16 mg) in 1 ml acidified water (pH = 5.5) slowly added. The reaction was allowed to proceed at room temperature for 20 hours. During the first hour, the pH was kept at 5.5. After termination of the reaction, 3 ml 1 mol/l hydroxylamine hydrochloride solution was added to destroy any excess of carbodiimide. The mixture was allowed to stand one hour, and was then dialysed first against 0.01 mol/l NaOH to remove uncoupled cortisol-3-(O-carboxymethyl)oxime, then against distilled water overnight. The product was lyophilised and stored at – 20 °C until required.

Mixed anhydride reaction

Here, 0.1 μmol bovine thyroglobulin subunit (16 mg) was dissolved in 1 ml saturated $NaHCO_3$-solution, pH = 8.5. 10 μmol cortisol-3-(O-carboxymethyl)oxime (4.4 mg) dissolved in 1 ml dioxane were mixed with 20 μmol triethylamine (2 mg) and cooled to 5 °C; then 11 μmol *iso*butylchloroformate (1.5 mg) were added and stirred for 20 min at 5 °C, to form the mixed anhydride. The cooled bovine thyroglobulin solution is added dropwise over 30 minutes and the reaction mixture allowed to stand at room temperature overnight. Removal of unreacted small molecules was carried out by dialysis as described above.

Antibody production

Ten rabbits of no fixed race or sex, aged 3 months were used. After 20 weeks, 7 had either died or had been bled out. The remaining 3 were boostered together with 3 new rabbits over a further 9 month period.

The lyophilised antigen was dissolved in physiological saline so that each animal received 0.016 μmol coupled bovine thyroglobulin subunit in 0.25 ml. An equal volume of complete *Freund*'s adjuvant was used to form a water in oil emulsion which was injected intradermally in 30–40 sites on the flanks and along the spine. The booster scheme and antibody-titer-curves are shown in figure 6. With the first two injections, each rabbit received 0.25 ml *Pertussis* vaccine as additional stimulus (2). Ten to fourteen days after the third and subsequent injections, blood was taken from an ear vein and tested for antibodies as follows: The sera were diluted with phosphate buffer and 100 μl portions incubated for 75 min with 100 μl tracer. 50 μl carrier serum and 1000 μl 125 g/l or 150 g/l polyethyleneglycol were used to separate bound and free tracer (5). Titer was defined as the serum dilution which bound 50% of the added tracer under assay conditions.

Production of a ^{125}I-cortisol tracer

The so-called 2-step method of tracer production is preferred. As cortisol is not easily directly iodinated, an easily iodinatable molecule is first reacted with the cortisol-3-(O-carboxymethyl) oxime, then labelled in a second step.

In view of the disadvantages of the carbodiimide reaction, a mixed anhydride method was used to couple either tyrosine-methyl-ester or tyramine to cortisol-3-(O-carboxymethyl)oxime in a molar ratio of 2:1. The reactants are dissolved in dry pyridine and the reaction carried out as described above. After completion of the reaction, the products are freed from solvent and the remaining substance dissolved in ethyl acetate and washed with 0.01 mol/l NaOH and 0.01 mol/l HCl. After removal of the ethyl acetate under vacuum distillation, the dry powder, either cortisol-3-(O-carboxymethyl)oxime-tyrosine methyl ester or -tyramine, was stored at – 20 °C until use. Before labelling, 1 mg of derivate was dissolved in 1 ml dioxane, and 4 ml 0.5 mol/l phosphate buffer pH = 7.5 added dropwise.

The labelling was carried out as follows:

20 μl steroid derivative (ca. 4 μg),
20 μl $Na^{125}I$ (2 mCi = 75 kBq) and
20 μl Chloramine T (10 μg)

were added to a siliconised plastic tube and allowed to react for 2 min, when the reaction is stopped by addition of 40 μl $Na_2S_2O_5$ (80 μg).

A pre-purification step is carried out as published (6). 100 μl distilled water and 200 μl ethyl acetate are added to the reaction tube and shaken; the organic phase is then transferred to a thin-layer chromatography plate. The aqueous phase is washed twice with ethyl acetate, and the organic phase transferred each time to the plate. The plate is then developed using the solvent system previously described (ethyl acetate: acetic acid: water – 9 + 1 + 0.1 by volume). After separation the plate is cut into thin strips and counted in a gamma counter. The strips with high radio-activity were eluted with ethyl acetate, the solvent then removed and the remaining substance dissolved in buffer (0.1 mol/l citrate buffer, pH = 4, containing 2.5 g/l gelatin and 2.5 g/l bovine serum albumin, both steroid free). Each fraction was tested for maximum binding (B_0) and unspecific binding (N) in antibody excess. The fractions with $B_0 > 90\%$ and $N < 5\%$ were pooled. If no fraction had these properties, the fractions with the highest binding were re-extracted into ethyl acetate and rechromatographed. Useable tracer was diluted to a concentration of ca. 2×10^8 counts/min · l (3.3 MBq/l) and stored at – 20 °C until use.

Radioimmunoassay

Cortisol-free serum

All standards were made up in cortisol-free serum or in a protein solution containing human albumin, and β and γ-globulins in physiological concentrations, in which each component had been tested for cortisol content. Dexamethasone-suppressed patients donated serum which was read off a buffer curve. Those patient sera showing no detectable cortisol were pooled and used. The "synthetic serum" was only used as an emergency measure, or in experimental situations. Standard curves made up in hormone-free serum and in the protein solution were identical.

Tracer-binding kinetics

The tracer binding of the antiserum K9 for the zero and 15 μg/dl (414 nmol · liter^{-1}) standard at room temperature shows a plateau after one hour. As the assay was almost in equilibrium, an assay time of 75 minutes was chosen. The results with 4 °C incubation were similar, but with lower binding.

pH 4-assay

The maximum affinity of cortisol binding globulin (CBG) for cortisol is seen at about pH 8. Under both acid and alkaline conditions, the affinity of CBG falls off rapidly, and at pH 4, CBG has no measurable affinity for cortisol (7).

Figure 2 shows the pH-dependance of the cortisol binding to the antibody K9. At pH 4 the affinity of the antibody is maximal, thus allowing an assay to be developed without prior denaturation of CBG, an advantage in the question of automation.

Albumin, as well as CBG, binds cortisol (8) and so the effect of albumin upon the accuracy of the assay at pH 4 was tested. As only 3 μl serum was used in the assay (see below) amounting to about 125 μg albumin, a significant quantity when compared

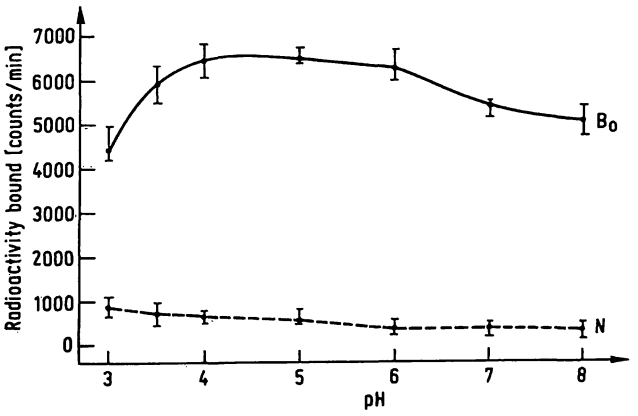

Fig. 2. pH-dependence of the antiserum K9 with maximal binding (B_0) and unspecific binding (N).

with that in the tracer (250 µg/100 µl), concentrations of 10–160 g/liter (1000–16000 µg/sample) were tested. There was no significant change in the binding of tracer to the antibody at pH 4.

Affinity studies

The affinity of the antisera was tested under assay conditions with the help of a *Scatchard* plot (see below), which after transformation of the standard curve data allows the affinity constant of the antibody for the antigen to be calculated. Figure 3 shows the *Scatchard* plot for antibody K 9 and gives an affinity constant of 0.11×10^{10} l/mol.

Optimised double antibody technique

A technique using combined second antibody (2. Ab) and polyethylene glycol in low concentrations has been developed in this laboratory (9) for peptide hormones; it accelerates the formation of precipitable 1.Ab–2.Ab complexes, which can either be centrifuged or filtered to effect a bound/free separation. The application of this method to the cortisol-RIA leads to a time saving of 50%, as well as an elevation of B_0, and reduction of N when the 2.Ab polyethylene glycol solution is added in 0.07 mol/l phosphate buffer pH 7.4.

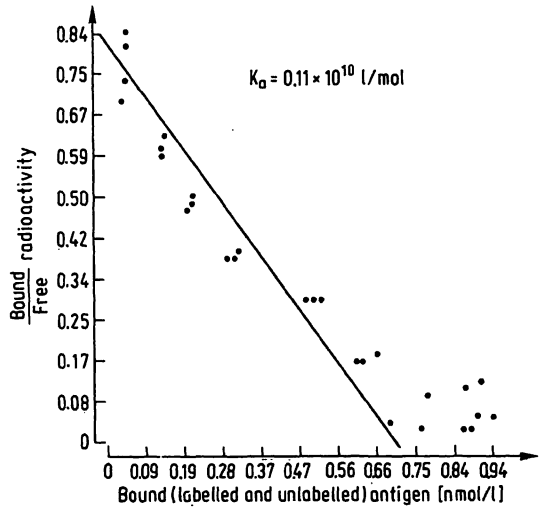

Fig. 3. *Scatchard* plot under assay conditions showing affinity constant of the antiserum K9. $K_a = 0.11 \cdot 10^{10}$ l/mol.

Tab. 1. Assay details.

Standard solution or serum	3 µl
[^{125}I]cortisol tracer in 0.1 mol/l Na-citrate-buffer pH=4 + 2.5 g/l gelatin + 2.5 g/l bovine serum albumin (ca. 10000 counts/min · 150 µl)	150 µl
Cortisol-antiserum (1:8000 in 0.1 mol/l Na-citrate buffer pH=4)	100 µl
75 min incubation at room temperature	
400 µl second antibody (lot-dependent) for each one hundred samples in 50 ml 60 g/l polyethylene glycol in 0.06 mol/l Na-phosphate buffer pH=7.4 + rabbit-γ-globulin (7 µg/sample)	500 µl
60 min incubation at room temperature	
Separation using filtration or centrifugation (10 min at 3000 *g*)	
Counting time 2 min	

Automation

The assay, as shown in table 1, lends itself for use in the automatic system developed in this department (10). Details of this automation have already been published (11). In brief, the capacity of the system is limited by the dilutor which can process 170–250 samples/hour with a coefficient of variation of < 1% for this assay.

Data processing

Figure 4 shows a typical standard curve using data for the automated assay for cortisol, and a spline-function (12). The curve form was characterised by use of the 50% intercept and the precision by the lower limit of detection.

Results

Synthesis of a pure cortisol-3-(O-carboxymethyl)oxime

Figure 5 shows an IR-spectrogramme of cortisol, testosterone and cortisol-3-(O-carboxymethyl)oxime. The arrows show the carbonyl groups at ca. 1710 cm^{-1} and ca. 1640 cm^{-1} in cortisol and only at 1640 cm^{-1} in testosterone which only has a C_3-keto group. In the case of cortisol-3-(O-carboxymethyl)oxime, the band at 1710 cm^{-1} due to the C_{20}-keto group is fully intact, whereas that at 1640 cm^{-1} has disappeared, showing a specific conversion of the C_3-keto group.

The keto group at C_3 appears to be more reactive, as the keto group at C_{20} is sterically hindered by the $C_{17,21}$-hydroxymethyl groups. This reaction rate difference is not seen at higher temperatures.

The effect of temperature on the rate and specificity of the reaction was studied at (a) 50–60 °C, (b) 10–15 °C and (c) room temperature (20–22 °C). In the case of (a) above, the reaction was complete within 60 min, but the specificity was poor, equimolar amounts of C_3- and C_{20}-derivatives being seen, together with unidentifiable

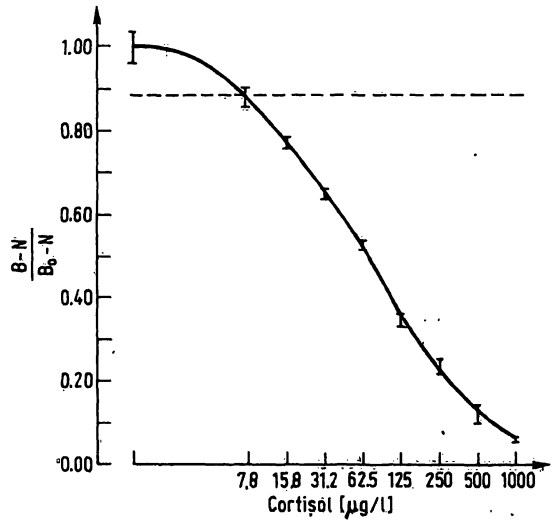

Fig. 4. Typical standard curve using antiserum K9, own [^{125}I]cortisol-3-(O-carboxymethyl)oxime-tyrosine-methyl-ester tracer and second antibody/polyethylene glycol-separation.

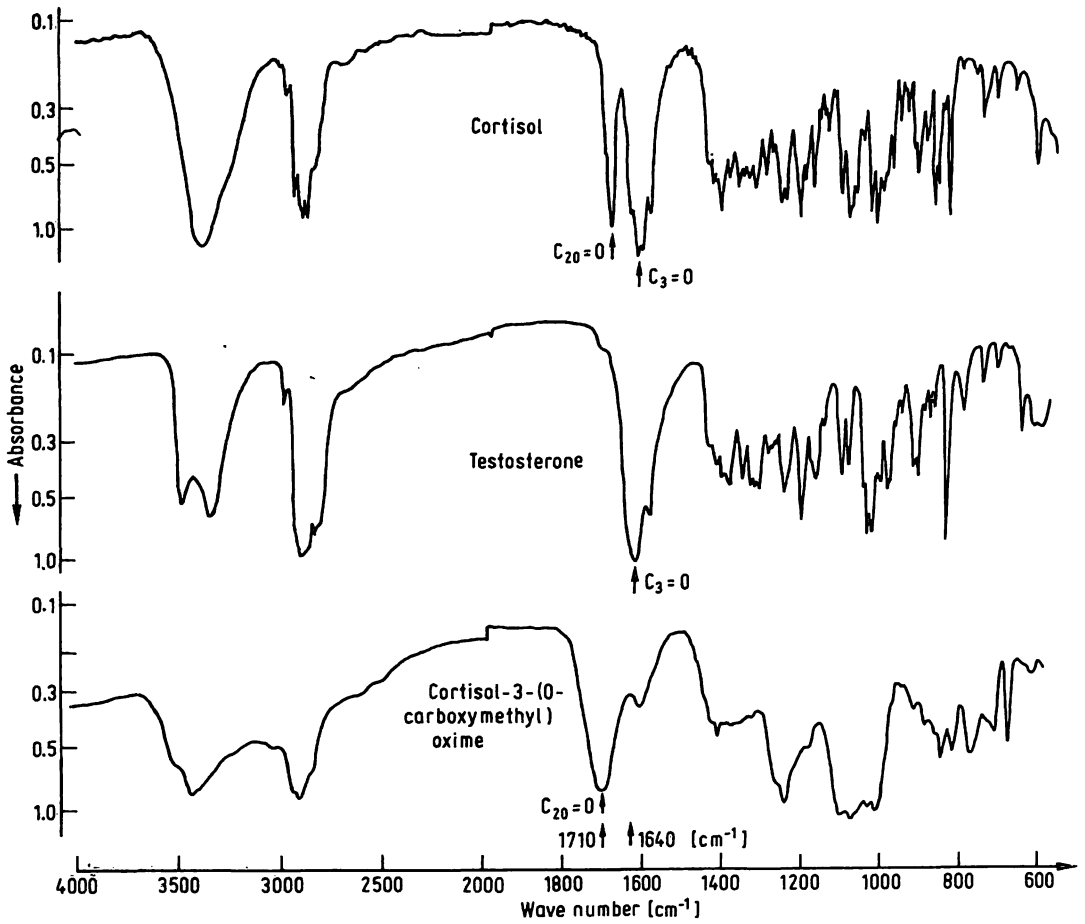

Fig. 5. Infrared-spectra showing absorption bands of the C_{20}- and C_3-keto groups. Above: cortisol, middle: testosterone, below: cortisol-3-(O-carboxymethyl)oxime.

side-products. At lower temperatures as in case (b), the reaction took two days and showed no better selectivity than at room temperature (case (c)), where the reaction was complete within 6 hours.

Antigen synthesis

The degree of coupling of cortisol-3-(O-carboxymethyl) oxime to the bovine thyroglobulin was determined by using [^{3}H]cortisol-3-(O-carboxymethyl)oxime and measuring the ratio of radioactivity in the starting material to that in the product.

Specific coupling of cortisol-3-(O-carboxymethyl)oxime to bovine thyroglobulin subunit was 5:1 (mol/mol) and unspecific coupling 2:1 using the carbodiimide method, the corresponding figures for the mixed anhydride method being 20:1 and 2:1, respectively.

A further disadvantage of the carbodiimide method is that it takes place at a slightly acid pH were the solubility of cortisol-3-(O-carboxymethyl)oxime is very low. It is possible that here, instead of a carboxyl group activation, a guanidine derivative is formed. It is also possible that the bovine thyroglobulin is partly polymerized and thereby denatured by carbodiimide reaction products (1). This is reflected by the poor water solubility of bovine thyroglobulin-cortisol-3-(O-carboxymethyl)oxime formed by the carbodiimide reaction.

Antibody production

Figure 6 shows the antibody titer curve over 42 weeks for a rabbit injected with the cortisol-3-(O-carboxymethyl)oxime complex formed by the carbodiimide reaction. Because of the low titer, all rabbits were boostered with the cortisol-3-(O-carboxymethyl)oxime complex formed by the mixed anhydride method. After a single injection the titer rose from 1:3200 to 1:16000 (K 2). The cortisol content of the antiserum at the concentration used in the assay was tested after pepsin-HCl digestion to destroy the antibody. The value lay under the detection limit of the assay and therefore proved to be unimportant.

Production of a [^{125}I]cortisol tracer

In the radioimmunoassay in current use in this laboratory (see below) a 50% intercept of 10 µg/dl (276 nmol · liter^{-1}) and less was achieved using the ^{125}I-cortisol-(O-carboxymethyl)oxime-tyrosine-methyl-ester derivative and a 50% intercept of 50 µg/dl (1380 nmol · liter^{-1}) for the [^{125}I]cortisol-(O-carboxymethyl)oxime-tyramine

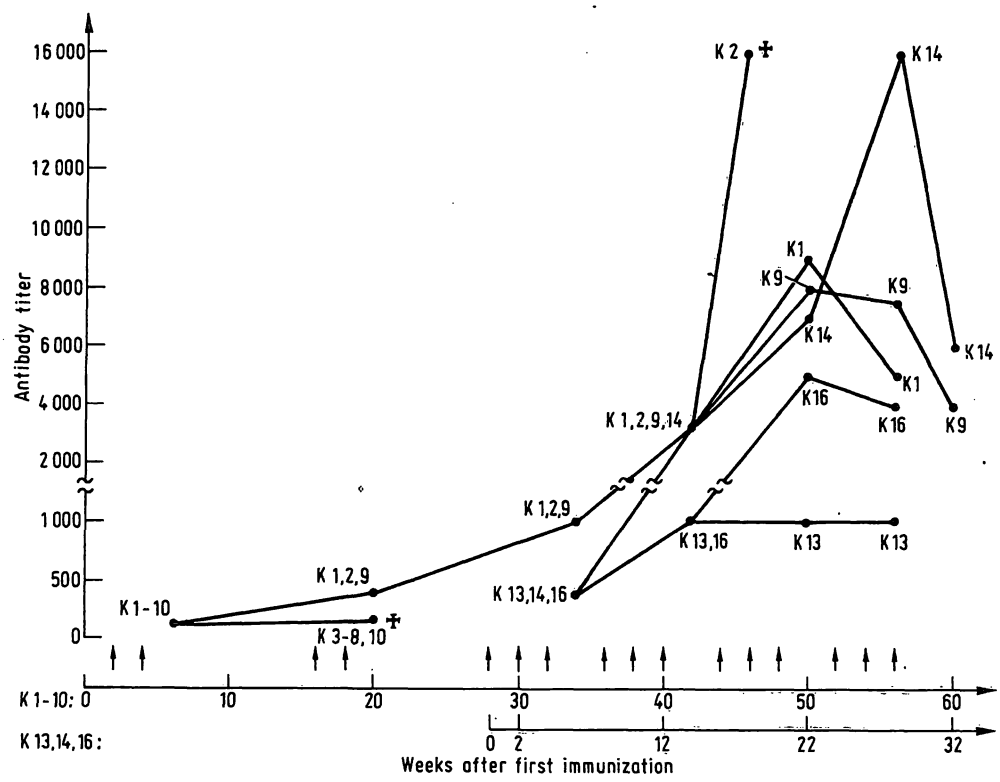

Fig. 6. Antibody-titer from rabbits injected with cortisol-3-(O-carboxymethyl)oxime-thyroglobulin-conjugates.
+ = rabbits died. From week 0–28 K1–K10 received a conjugate cortisol-3-(O-carboxymethyl)oxime: bovine thyroglobulin subunit = 4:1, from the 28th week a conjugate cortisol-3-(O-carboxymethyl)oxime: bovine thyroglobulin subunit = 6:1 using carbodiimide reaction. Rabbits 13, 14, 16 received only the latter conjugate (week 28 = 0). From week 16 K 13, 14, 16 and from week 44 K 1, 2, 9 received a conjugate cortisol-3-(O-carboxymethyl)oxime: bovine thyroglobulin subunit = 20:1 using the mixed anhydride method.

derivative. The tyrosine-methyl-ester derivative was introduced for reasons of sensitivity. This difference in the form of the standard curve with these derivatives agrees with already published data (6). The shelf-life of the tracer is at least several weeks, thus making labelling necessary about 6 times a year.

Radioimmunoassay

Correlation studies

The performance of antiserum K9 was compared with the antiserum in current use in the laboratory, which had been donated by Professor *E. Kuss* (I. Frauenklinik der Universität München). The correlation was studied in 50 routine sera measured with both antisera on the same day using identical reagents. The regression data for the line $y = a + bx$ where y represents the K9 values and x the "control-method", was excellent with $a = 3.4$ µg/l and slope $b = 0.99$. The correlation coefficient (r) was 0.94 and gave a p-value of < 0.001, i.e. highly significant.

A correlation with the ^{125}I-tracer using the antiserum donated by *Kuss* and pH=4-assay compared with the earlier ^{3}H-assay with extraction and column chromatography separation of bound and free antigen (13) gave a correlation coefficient for 43 data pairs of $r = 0.87$ and for the regression line $a = 10.6$ µg/l and $b = 0.971$. Again the correlation was highly significant. These results show that the new assay with antiserum K9 gives the same results as the antiserum donated by *Kuss* using ^{125}I or ^{3}H (an indirect, but logically grounded assumption).

Specificity

Figure 7 shows the cross-reaction profile of both other steroids using antiserum K9. The cross-reaction of cortisol is set at 100% and the cross-reaction of the steroids is by weight (mean of several concentrations). As a comparison, the antiserum donated by *Kuss* cross-reacted with cortisone to an extent of 85%, 11-deoxycortisol 30% and prednisolone 35%.

Quality control

The following results were obtained using the described assay procedure partly with antiserum K9 and partly with antiserum donated by *Kuss*.

The intra-assay and inter-assay coefficients of variation (C.V.) were tested. The intra-assay C.V. for 5 sera reflects the parabolic form of a precision profile with high C.V. at low and high concentrations, i.e. where the standard curve is flattest (11).

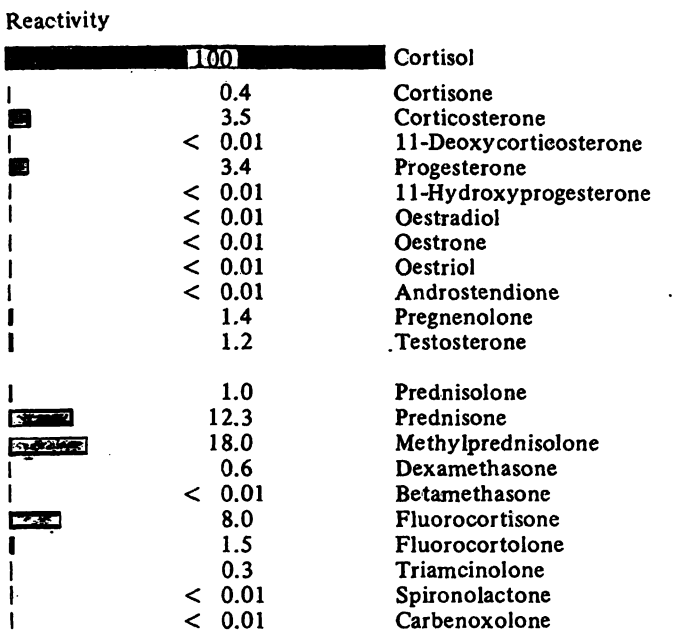

Fig. 7. Shows cross-reactivity of antiserum K 9 with several naturally occurring and synthetic steroids.

The inter-assay C.V. for 33 consecutive assays at a level of 174 µg/l (480 nmol · liter^{-1}) was 10.5%. The mean recovery of added cortisol to a patient serum in the range of 10–500 µg/l (28–1380 nmol · liter^{-1}) was 95 ± 7.2% for 15 different concentrations.

Normal range

Due to the circadiurnal rhythm of serum cortisol levels, all blood was taken at 9 a.m. under fasting conditions. The normal range for 30 patients lay between 55 and 215 µg/l (151–593 nmol · liter^{-1}) with a mean and standard deviation of 143 ± 55 µg/l. The distribution of these 30 sera shows a non-*Gaussian*-distribution. To prove the distribution type at least 100 sera must be measured.

Discussion

Being unsuccessful in repeating published methods for the production of "pure" cortisol-3-(O-carboxymethyl) oxime, and because of the obscure criteria for determining structure and purity (14, 15, 16), a new start was made (17). By optimising the synthesis in the absence of water and under pyridine catalysis, together with careful choice of the chromatographic solvent, it was possible to prepare a cortisol-3-(O-carboxymethyl) oxime of satisfactory purity in thin-layer chromatography, with a yield of about 70% of the theoretical. Infra-red spectroscopy showed that only the C-3-isomer of cortisol-(O-carboxymethyl)oxime was produced (fig. 5). This coupling-method can be used with minor modifications for all steroids with a C_3-keto group and a non-aromatic A-ring. For testosterone, for example, with only a C_3-keto group, the synthesis is much easier than for cortisol.

Further attempts to produce a direct cortisol-3-tyrosine derivative by reductive amination (*Leuckart-Wallach*) met with no success. The use of sodium cyanoborhydride ($NaBH_3CN$) as a specific reductant for azomethines (18) in the presence of H^+ was only successful for the reduction of cortisol-3-(O-carboxymethyl)oxime.

The use of the mixed anhydride method for coupling cortisol-3-(O-carboxymethyl)oxime to bovine thyroglobulin is, because of the alkaline conditions, to be prefered for steroid conjugation; the acid solutions needed for the carbodiimide conjugation create solubility problems. This was plainly seen in the boostering of rabbits with the mixed anhydride product in rabbit K9 which led to an immediate 5-fold increase in the antiserum titer. It is well known that there is a minimum ratio of hapten to carrier, below which no or only a poor antibody response is obtained (19). For steroids, a mixed anhydride reaction combines an optimum pH/protein solubility conditions with the maximum incorporation.

That the tracer is stable for at least two months means that the somewhat complicated methodology must be carried out at the most 6 times a year, or when larger amounts of tracer can be made, even less frequently. Although a two-phase system was used in the labelling, it should be possible to carry out the labelling in an organic phase using a soluble iodination agent such as Iodogen® (20), an alternative would be labelling of the tyrosine-methyl-ester with subsequent coupling to the cortisol-3-(O-carboxymethyl)oxime using the mixed-anhydride method under water-free conditions. This would be less harsh as no oxidation agent would come into contact with the cortisol-3-(O-carboxymethyl) oxime. The disadvantage is the longer reaction time (about 14 hours), in which radiolysis could give rise to more damage than the Chloramine-T or Iodogen.

The attempt to simplify the purification of tracer, by using Sephadex LH-20 gave no satisfactory results.

No figure for the specific activity of the tracer could be given, because of the uncertainty of the recovery through the many purification steps.

The use of a pH=4-assay (11) allowed full automation with a precision in the steep-part of the standard curve of under 5% as measured by the intra-assay C.V.

The antiserum K9, produced with the pure cortisol-3-(O-carboxymethyl)oxime-bovine thyroglobulin conjugate, showed excellent specificity, and gave rise to a sensitive, precise, accurate and rapid assay. This assay could be run using either standards in cortisol-free serum or in a carefully prepared "synthetic serum" to give the same results as the assays previously in use in the laboratory. This antiserum with its lower cross-reactivity to

other steroids has potential advantages, especially in certain scientific problems.

The development of a direct and homologous assay for serum cortisol has led to a time and work-saving of above 75% in comparison with the original ^{3}H-method with extraction and column chromatography (13). These points are very important, especially in a laboratory rationalisation programme, where a simple, short assay is preferable to a long and complicated one.

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